DIFFERENTIAL ACTIVITIES OF THREE FAMILIES OF SPECIFIC β(1,3)GLUCAN SYNTHASE INHIBITORS IN WILD-TYPE AND RESISTANT STRAINS OF FISSION YEAST.

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Running title: Differential activities of glucan synthase inhibitors

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Three specific β(1,3)glucan synthase (GS) inhibitor families -papulacandins, acidic terpenoids and echinocandins - have been analyzed in *Schizosaccharomyces pombe* wild-type and papulacandin-resistant cells and GS activities. Papulacandin and enfumafungin produced similar *in vivo* effects, different to that of echinocandins. Also, papulacandin was the strongest *in vitro* GS inhibitor (IC_{50} 10^3-10^4-fold lower than with enfumafungin or pneumocandin), but caspofungin was by far the most efficient antifungal since it was 1) the only drug that affected resistant cells (MIC close to that of the wild-type); 2) a strong inhibitor of wild-type GS (IC_{50} close to that of papulacandin); and 3) the best inhibitor of mutant GS. Moreover, caspofungin showed a special effect for two GS inhibition activities, of high- and low-affinity, separated by two log-orders, with no increase in inhibition. *pbr1-8* and *pbr1-6* resistances are due to single substitutions in the essential Bgs4 GS, located close to the resistance hot spot 1 region described in *Saccharomyces* and *Candida* Fks mutants. Bgs4^{pbr1-8} contains the E700V change, four residues N-terminal from hot spot 1 defining a larger resistance hot spot 1-1 of 13 amino acids. Bgs4^{pbr1-6} contains the W760S substitution, defining a new resistance hot spot 1-2. We observed spontaneous revertants of the spherical *pbr1-6* phenotype and found that an additional A914V change is involved in the recovery of the wild-type cell shape but maintaining the resistance phenotype. A better understanding of the mechanism of action of the antifungals available should help to improve their activity and to identify new antifungal targets.

The number of antifungal families available and their use in therapy is very limited (1-4). Recently, a new family of specific fungal cell wall synthesis inhibitors has emerged as an alternative antifungal therapy and is gaining increasing relevance yearly (5-7).

The cell wall is a structure external to the plasma membrane and is present in all fungal cells. Its integrity is crucial and it constitutes the exoskeleton that confers mechanical strength and osmotic resistance to fungal cells (8-10). In mammalian cells, the cell wall is absent and consequently drugs that interfere with its synthesis are attractive as potential antifungal agents. β(1,3)glucan is a major contributor to the framework of the cell wall. There are several families of antifungal drugs whose mode of action is not well known, although they clearly interfere with β(1,3)glucan synthesis by inhibiting the β(1,3)glucan synthase (GS) enzyme. These inhibitors include echinocandins (lipopeptides), papulacandins (glycolipids) and acidic terpenoids such as enfumafungin (2,11). To date, only the echinocandins caspofungin, micafungin and anidulafungin have been approved (in years 2002, 2005 and 2006, respectively) for treatment of invasive fungal infections (1,3,5,7,12).

The fission yeast *Schizosaccharomyces pombe* provides an appealing model for studies addressing cell wall synthesis and morphogenesis. The *S. pombe* cell wall has no detectable chitin (10) but it contains three different essential β-glucans: a branched β(1,3)glucan, which is the major contributor to the cell wall structure; a minor linear β(1,3)glucan, concentrated in the primary septum, with minor amounts in the cell wall, and a minor branched β(1,6)glucan (13,14). *S. pombe* contains four essential putative GS catalytic subunits: Bgs1 to Bgs4. Bgs1 is responsible for the synthesis of the linear β(1,3)glucan and

primary septum. Bgs2 is essential for spore wall maturation, and Bgs3 is essential, although its function remains unknown. Bgs4 is the only subunit that has been shown to form part of the GS enzyme. It is responsible for the major part of cell wall β(1,3)glucan synthesis and *in vitro* GS activity, and it is essential for the maintenance of cell integrity during cell growth and separation (14-17). The different essential functions of Bgs proteins in cell morphogenesis make them good targets for the study of antifungal drugs that specifically inhibit β(1,3)glucan synthesis.

The *S. pombe* Bgs family is homologous to fungal Fks and plant CalS proteins, considered to be putative GS catalytic subunits (8,18). Fungal resistance to GS inhibitors is clearly associated with mutations in conserved short regions (hot spots) of the Fks proteins, indicating that this mechanism is well conserved in fungi (12,19,20). In addition, intrinsic echinocandin-resistant fungi contain natural substitutions in the conserved Fks region that are determinants of their resistance (19,21-23). Most of the mutants resistant to GS inhibitors have been isolated as resistant to echinocandins. Only some *Saccharomyces cerevisiae* and *S. pombe* mutants have been selected as resistant to papulacandin (24,25), in each case defining a single complementation group called *pbr1*. However, whereas the *S. cerevisiae pbr1-1* mutation has been associated with *FKS1*, the *S. pombe pbr1*$^+$ gene remains unknown.

In light of the above data, we were prompted to study and compare the mechanism of action of the three antifungal families: papulacandins, acidic terpenoids and echinocandins. Using *S. pombe* as a model, we examined the *in vivo* and *in vitro* effect of the antifungals on cells and the GS of wild-type and resistant mutants. Our data point to important differences among the antifungal families in both cells and GS activity. Caspofungin was overall the best inhibitor of cells and GS tested, not only of the wild type but also resistant cells. Although *S. pombe* vegetative cells contain three essential Bgs subunits, the antifungal resistance is exclusively associated with Bgs4, suggesting that Bgs1 and Bgs3 are intrinsic resistant subunits. Papulacandin selection afforded two new amino acid substitutions, expanding the resistance hot spot 1 to 13 residues and defining a new resistance hot spot 1-2. These new sites, which are important for resistance and interaction with antifungals, should help to understand the mechanism of action of antifungals and the resistance mechanism of the Bgs / Fks proteins.

EXPERIMENTAL PROCEDURES

Strains and culture conditions. The *S. pombe* strains used were isogenic to wild-type strain h$^-$ 972. The *pbr1-1*, *pbr1-2*, *pbr1-3*, *pbr1-6* and *pbr1-8* mutants were obtained by ethyl methanesulfonate mutagenesis (15-30 % survival) and selection in the presence of 20 μg/ml of papulacandin B (25). The five mutants were backcrossed three times with the wild-type strain. In all the cases, tetrad analysis revealed a 2R:2S segregation, indicating the monogenic trait or the resistance phenotype. The dominant / recessive analysis was performed in stable heterozygous diploid strains, using the mat2P-B102 mutation (26). All the papulacandin resistance mutations proved to be recessive. Complementation analysis was carried out in diploid strains and showed that the five resistance mutations are alleles of the same gene, which was named *pbr1* (from papulacandin B resistant).

The standard complete yeast growth medium (YES), selective medium (EMM) supplemented with the appropriate amino acids and sporulation medium (SPA) (26,27) have been described previously. Cell growth was monitored by measuring the A_{600} of early log-phase cell cultures in a Coleman Junior II spectrophotometer (OD_{600} 0.1 = 1x10^7 cells/ml). The general procedures for yeast and bacterial culture and genetic manipulations were carried out as described (27,28).

Plasmids and DNA techniques. The multicopy pAL-*bgs4*$^+$ (*S. cerevisiae LEU2* selection) and integrative single copy pJK-*bgs4*$^+$ plasmids (*S. pombe leu1*$^+$ selection) have been described elsewhere (15). All the *bgs4*$^+$ plasmids contain an 8.84 kb *Pst*I-*Nhe*I *bgs4*$^+$ fragment. The integrative phis3-*bgs4*$^+$ plasmid (*his3*$^+$ selection) contains the Bluescript KS$^+$ backbone, the 2.3 kb *Eco47*III-*Dra*I *his3*$^+$ sequence cloned into *Eco*RV (*Kpn*I-*Sac*I orientation), and the *bgs4*$^+$ sequence from pAL-*bgs4*$^+$ cloned into *Pst*I-*Not*I. p81X-*bgs4*$^+$ is pJR2-81XL (*LEU2* selection and 81X version of the thiamine-repressible *nmt1*$^+$ promoter) (29) containing the *bgs4*$^+$ ORF (15).

pAL-*bgs4*$^{pbr1\text{-}8}$ contained *bgs4*$^{pbr1\text{-}8}$ obtained from the *pbr1-8* strain by plasmid gap-repair. pAL-*bgs4*$^+$ was digested with *Sac*I (removing the *bgs4*$^+$ ORF, 680 bp of promoter and 188 bp of terminator), transferred into strain *pbr1-8*, and

repaired pAL-*bgs4*$^{pbr1\text{-}8}$ plasmids were recovered from transformants. Integrative pJK-*bgs4*$^{pbr1\text{-}8}$ and phis3-*bgs4*$^{pbr1\text{-}8}$ and regulatable p81X-*bgs4*$^{pbr1\text{-}8}$ contained *bgs4*$^{pbr1\text{-}8}$ from pAL-*bgs4*$^{pbr1\text{-}8}$. The 8.8 kb region of *bgs4*$^{pbr1\text{-}8}$ contained a single base substitution, A2099T, resulting in the E700V amino acid change of Bgs4, and *bgs4*$^{pbr1\text{-}6}$ contained the single G2279C base substitution, resulting in the W760S amino acid change of Bgs4 (see results).

bgs4$^{pbr1\text{-}8}$ gene deletion was performed in a *pbr1-8* / *pbr1-8* diploid by removing the entire ORF of a *bgs4*$^{pbr1\text{-}8}$ copy, as described for *bgs4*$^{+}$ gene deletion (15). Bgs4 is essential and therefore haploid *bgs4*$^{pbr1\text{-}8}\Delta$ deletion strains were maintained viable with a plasmid expressing *bgs4*$^{+}$.

Antifungal drugs and resistance assays. The antifungals were generous gifts from Ciba-Geigy / Novartis (papulacandin B), Tokyo Jozo (aculeacin A) and Merck, Sharp and Dohme (enfumafungin, pneumocandin B0 and caspofungin). The antifungals were kept at -20ºC in stock solution (10 mg/ml in DMSO, except caspofungin, which is highly soluble in water) and assayed at the final concentrations specified in the text and figures.

For micro-culture assays of large numbers of samples, late log-phase cultures grown in YES medium were diluted to a cell density of 2 x 10^6 cells/ml in 200 µl of YES medium containing increasing concentrations of antifungal (0, 1, 2, 5, 10, 20, 50 and 100 µg/ml) or an equivalent volume of solvent. The cell cultures were incubated in an orbital roller at 28ºC and turbidity was analyzed after 24 and 48 h of incubation, affording values ranging from 0 (-, no turbidity, wild-type cells in the presence of a lethal concentration of antifungal) to 100 (+++, total turbidity, wild-type cells in the absence of antifungal). The MIC (Minimal Inhibitory Concentration) was determined as the minimal concentration of antifungal that produced complete cell growth inhibition after 24 h of incubation. The values were calculated from at least three independent experiments.

Enzyme preparation and β(1,3)glucan synthase (GS) assay. Cell extracts and GS assays were performed essentially as described (30). Cell extracts were obtained from early log-phase cells grown in YES medium at 28ºC. The cells were washed with buffer A (50 mM Tris-HCl pH 7.5, 1 mM EDTA and 1 mM β-mercaptoethanol), suspended in 100 µl of buffer A containing 50 µM GTPγS to preserve enzyme activity, and broken with glass beads. Membrane enzyme extracts were resuspended in the same buffer containing 33% glycerol and 50 µM GTPγS and stored at -80ºC. All GS assays (150 µM GTPγS and 15-25 µg of protein) were carried out in duplicate and the values were calculated from at least three independent cell cultures.

Other procedures. The fractionation of cell wall polysaccharides, enzymatic lysis of cell suspensions (31), and fluorescence microscopy of cell walls stained with Calcofluor White (30), have been described previously.

RESULTS

The pbr1 mutants present increasing cell-wall related phenotypes. The isolation of *S. pombe* mutants resistant to papulacandin B, which defined a single complementation group designated *pbr1* (for papulacandin B resistant), has been reported previously (25). *pbr1-6* cells show a special spherical morphology (Fig. 1), which has been associated with a single recessive gene called *sph1-1* (24). This mutant was characterized as being defective in cell wall galactomannan, which could account for a cell integrity defect responsible for the spherical phenotype. In fact, osmotic stabilization with sorbitol suppressed that phenotype (Fig. 1A). However, the morphology of the other *pbr1* alleles was that of both normal and lemon-shaped cells (Fig. 1A and data not shown). This led us to consider the possibility that both phenotypes might be related, *pbr1-6* being the result of a more aggravated phenotype. Accordingly, the cell-wall related phenotypes of *pbr1-6* and *pbr1-8* (the latter being representative of the other mutant alleles) were examined (Table 1).

The fluorochrome Calcofluor White specifically binds to growing poles, and with especially high affinity for the septum (14). *pbr1-8* and *pbr1-6* cells showed increasing Calcofluor staining, which was more intense in *pbr1-6* cells, where the compound stained the whole cell wall and the septa appeared thicker and more strongly stained (Fig. 1A). Cell wall analyses also revealed increasing phenotypes: from the wild-type to *pbr1-8*, to *pbr1-6* (Table 1). With mild alkali extraction of the cell walls a gradual increase in susceptibility was observed. Lysis of cell suspensions with Novozyme also elicited increasing susceptibility. By contrast, the mutant cells exhibited increasing resistance to Zymolyase degradation. Novozyme degrades the

cell wall, suggesting an increasing defect in cell wall structure and integrity. However, Zymolyase does not contain α-glucanases and hence resistance to degradation indicates a progressive increase in cell wall α-glucan levels. This was confirmed by cell wall fractionation, which revealed a gradual increase in total cell wall, β-glucan and α-glucan, and a gradual decrease in galactomannan. Similarly, the amount of cell wall hexoses changed: glucose increased whereas mannose and galactose decreased (Table 1).

The above findings indicate that both *pbr1-8* and *pbr1-6* mutants show similar cell-wall-related phenotypes, more aggravated in *pbr1-6*. However, although the mutants were selected as being resistant to a β(1,3)glucan synthase (GS) inhibitor, the kinetic parameters of *in vitro* mutant GS activities (specific activity, Km and Vmax) were not altered either in the absence (24,25) or in the presence of papulacandin (50 μg/ml, data not shown).

Differential in vivo *effect of three families of specific β(1,3)glucan synthase (GS) inhibitors on wild-type and resistant stains.* The *pbr1* mutants were obtained as being resistant to papulacandin B and also showed resistance to aculeacin A. However, *pbr1-6* was again different since it showed normal sensitivity to aculeacin A. Owing to the different behavior of the *pbr1-6* and *pbr1-8* mutants with aculeacin A and to the fact that only a few antifungals have been analyzed, antifungals representative of the different families of specific GS inhibitors (2) were selected to study their *in vivo* and *in vitro* effects on wild-type and resistant strains and GS activities: papulacandin B (papulacandins), enfumafungin (acidic terpenoids), aculeacin A, pneumocandin B0 and caspofungin (echinocandins) (Fig. 2).

First, a different *in vivo* inhibitory capacity was observed, depending on the antifungal family. In wild-type cells, papulacandin and enfumafungin produced a dramatic and complete arrest of cell growth, whereas the echinocandins permitted residual cell growth even at higher concentrations (Fig. 3A). This was accompanied by a different lytic effect. Papulacandin and enfumafungin led to rapid and total cell lysis whereas the echinocandins produced lysed cells accompanied by swollen, round and aberrant cells (data not shown). Similar two-type antifungal effects were also observed with the *pbr1-8* and *pbr1-6* resistances; complete resistance and an unaltered morphology with papulacandin and enfumafungin, and weaker resistance and cells becoming round and aggregated with the echinocandins (Fig. 3A,B) or even no resistance at all, as in the case of *pbr1-6* strain with aculeacin (Fig. 3B).

The MIC of the three antifungal families was similar in the wild-type strain (5-10 μg/ml, Table 2) and again defined two antifungal groups in the resistant strains: highly resistant with papulacandin and enfumafungin, and less resistant with the echinocandins (Table 2). Like aculeacin, the other echinocandins also elicited differences between the resistant strains, *pbr1-8* exhibiting higher resistance than *pbr1-6* (Table 2).

Differential in vitro *effect of three families of specific GS inhibitors on wild-type and resistant GS activities.* Owing to the different effects observed *in vivo* with the antifungals analyzed and to the fact that all these antifungals are specific GS inhibitors, the effect of the three inhibitor families on the *in vitro* GS activity of the wild-type strain was analyzed (Fig. 4A). Enfumafungin and pneumocandin showed similar IC_{50} (half-maximal inhibitory concentration) values. Surprisingly, papulacandin showed a very strong GS inhibitory activity, with an IC_{50} 10^3-10^4-fold lower than that of enfumafungin and pneumocandin (Fig. 4A and Table 2).

Accordingly, the *pbr1-6* and *pbr1-8* mutants were analyzed for their *in vitro* GS resistance to the three antifungals (Fig. 4B and Table 2), and the results were compared to the data previously reported for aculeacin (25). Both mutant GS showed high resistance to all the antifungals, with an IC_{50} above 250 μg/ml even in the case of *pbr1-6* GS with aculeacin. However, some differences were observed between both mutant GS (Fig. 4B): 1) *pbr1-8* GS showed higher resistance to the inhibitors than *pbr1-6* GS, which at least correlates with the lower resistance to the echinocandins of the *pbr1-6* cells; 2) enfumafungin and pneumocandin promoted a specific activation of *pbr1-8* (110-120%) but not of *pbr1-6* GS activity; 3) *pbr1-8* GS resistance to papulacandin remained above that of *pbr1-6* across the range analyzed, whereas with enfumafungin and pneumocandin it decreased faster, lower resistance being observed at high drug concentrations. These results show that 1) each antifungal family produces a different effect on wild-type and mutant GS activities; 2) each mutant GS exhibits a different

resistance pattern, depending on the inhibitor analyzed.

Novel and special effect of caspofungin on in vitro *wild-type and resistant GS activities.* Owing to its clinical relevance, the effect of the echinocandin derivative caspofungin on wild-type GS activity was analyzed (Fig. 4C and Table 2). Caspofungin proved to be a potent GS inhibitor, with an interesting novel effect on its activity. First, the compound showed strong GS inhibitory activity, with an IC_{50} 10^2-10^3-fold lower than that of the antifungals tested, except papulacandin (IC_{50} still 10-fold lower). Second, caspofungin promoted two GS inhibition levels: a high-affinity inhibition, with a gradual decrease in GS levels down to 40% activity, and a low-affinity inhibition, with a new decrease in GS activity until complete inhibition. Both inhibition ranges were separated by a plateau of two orders of magnitude in which increasing caspofungin did not produce any additional inhibitory effect (Fig. 4C).

As observed with other antifungals, *pbr1-8* GS was more resistant to caspofungin than *pbr1-6* GS and showed a constant activation (110%) over a broad concentration range of inhibitor (Fig. 4D). *pbr1-8* GS proved to be resistant to the high-affinity inhibition but sensitive to the low-affinity inhibition. *pbr1-6* GS showed lower resistance, with partial inhibition in the high-affinity inhibition range, remaining constant during the plateau of two log orders of caspofungin. Increasing concentrations of caspofungin elicited a complete inhibition, similar to the wild-type inhibition. This indicates that *pbr1-8* and *pbr1-6* GS are only resistant to the high-affinity inhibition effect of caspofungin. In addition, caspofungin was a potent inhibitor of mutant GS since it was the only drug that produced measurable IC_{50} values, which were lower in *pbr1-6* than in *pbr1-8* GS (150 and 250 μg/ml, respectively; Fig. 4D and Table 2).

$bgs4^+$ is unable to suppress the pbr1-8 resistance phenotype. Previously, *pbr1-8* has been genetically linked to *cwg1-1*. Both are GS-related genes, suggesting that $pbr1^+$ and $cwg1^+$ could be the same gene. *pbr1-8* resistance is recessive, but cloning of the $pbr1^+$ gene has been unsuccessful, as is the case of $cwg1^+$ (25,32). Cloning from a cosmid clone of the $bgs4^+$ gene of the essential GS subunit revealed that $bgs4^+$ is allelic to $cwg1^+$ (15).

The above results also suggested that $pbr1^+$ and $bgs4^+$ could be the same gene. The fact that in *S. cerevisiae* and other fungi the resistance to GS antifungals is due to mutations in the Fks GS homologues (12,19,25,33-36) supports the idea that specific *bgs4* mutations could be responsible for the resistance phenotypes. To test this notion, the resistance phenotypes of *pbr1-8* and *pbr1-6* mutants expressing $bgs4^+$ (own promoter, multicopy and single-copy integrative plasmids) were analyzed. *pbr1-8* and *pbr1-6* resistances are recessive (25) (see below: Fig. 6B and Table 3). However, $bgs4^+$ was unable to suppress the papulacandin, enfumafungin and pneumocandin resistances of *pbr1-8*, although it did suppress the resistance to caspofungin (Fig. 5A and Table 3). This was not the case with *pbr1-6*, in which suppression of all the resistances by $bgs4^+$ was observed (Fig. 5B and Table 3), as would be expected if $pbr1^+$ and $bgs4^+$ were the same gene. A similar absence of *pbr1-8* suppression by $bgs4^+$ was observed in the *in vitro* GS resistance of *pbr1-8* cells expressing $bgs4^+$ (Fig. 5C). This effect was not due to a defective p$bgs4^+$ since no changes were found in the 8.8 kb $bgs4^+$ sequence and p$bgs4^+$ was able to suppress the phenotype of other *bgs4* mutants (15).

In order to study whether *pbr1-8* resistance was related to $bgs4^+$, *bgs4* from the pbr1-*8* strain ($bgs4^{pbr1\text{-}8}$) was cloned and analyzed. p$bgs4^{pbr1\text{-}8}$ did not confer resistance to a wild-type strain, as expected for a recessive mutation, but it did provide resistance to a *bgs4*Δ strain (Fig. 6A). Similarly, a heterozygous WT / *pbr1-8* diploid was sensitive but *bgs4*Δ / *pbr1-8* showed resistance to the GS inhibitors (Fig. 6B and Table 5). Contrary to the incapacity of $bgs4^+$ to suppress the haploid *pbr1-8* resistance, $bgs4^+$ was able to suppress the *bgs4*Δ / *pbr1-8* resistance. When a regulatable promoter was used, the *bgs4*Δ / *pbr1-8* strain showed resistance when $bgs4^+$ expression was repressed and the sensitive phenotype when $bgs4^+$ was induced (Fig. 6C). This suggested that $bgs4^{pbr1\text{-}8}$ is responsible for at least part of the resistance of *pbr1-8*.

Sequencing of the *bgs4* ORF and adjacent regions of the *pbr1* mutant alleles revealed the substitution of only one base in the 8.8 kb DNA sequence of each mutant. *pbr1-8* and the rest of the alleles, except *pbr1-6*, contained the same A2099T substitution, resulting in the E700V amino acid change. *pbr1-6* contained the G2279C substitution, encoding the W760S amino acid change; 60 amino acids C-terminal from the *pbr1-8* mutation. Both residues -E700 and W760- are very well conserved and are located in the predicted first transmembrane

region of the Bgs / Fks / CalS protein family (Fig. 7A). Interestingly, the Bgs4^{pbr1-8} mutation is close to a conserved 9-amino-acid resistance hot spot 1 described from the Fks sequences of *Saccharomyces* and *Candida* species, whose mutations confer resistance to echinocandins (Fig. 7B) (12,21,22,37-43). The Bgs4^{pbr1-8} mutation permitted a larger 13-amino-acid hot spot 1-1 to be defined. In addition, the Bgs^{pbr1-6} mutation was located in a new resistance site, C-terminal from the hot spot 1-1, defining a new hot spot 1-2 of resistance to papulacandins, enfumafungin and echinocandins (Fig. 7B).

To confirm that the Bgs4 E700V and W760S mutations were responsible for the *pbr1-8* and *pbr1-6* resistances, plasmids with the corresponding *bgs4* substitution were made by site-directed mutagenesis and analyzed in a *bgs4*Δ strain. The Bgs4^{pbr1-8} and Bgs4^{pbr1-6} protein levels were similar to that of Bgs4 (data not shown). Both *bgs4* mutants conferred the same resistances as those of the *pbr1-8* and *pbr1-6* strains. In addition, *bgs4*$^{pbr1-6}$ generated a spherical phenotype similar to that of the *sph1-1* mutant (Fig. 1A), indicating that *sph1*$^{+}$ is also allelic to *bgs4*$^{+}$ and that both *sph1-1* and *pbr1-6* phenotypes are due to the single W760S mutation. The spherical *pbr1-6* (*sph1-1*) phenotype was unstable, originating spontaneous morphological revertants with an elongated cell shape and faster growth. Sequencing of the *bgs4*$^{pbr1-6}$ revertants revealed the initial W760S mutation and a spontaneous A914V suppressor mutation (C2741T base change) (Fig. 1B). The suppressor function of A914V was confirmed by inserting this mutation into p*bgs4*$^{pbr1-6}$ and observing a similar suppression (Fig. 1B). However, the antifungal resistance was not affected (Table 3), indicating that the A914V suppressor is specific to the *pbr1-6* morphological and cell integrity defects.

Genetic study of the absence of pbr1-8 suppression by bgs4^{+}. The fact that the resistance of the haploid *pbr1-8* strain was not suppressed but the resistance of the *pbr1-8* / *bgs4*Δ strain was suppressed by *bgs4*$^{+}$ suggested the possibility of a second recessive resistance mutation. This second resistance should be linked to *bgs4*$^{pbr1-8}$ since the resistance segregated 2R:2S in genetic crosses.

To test this, larger DNA fragments containing *bgs4*$^{+}$ and adjacent ORFs were cloned. None of the fragments suppressed the *pbr1-8* phenotype but they did suppress that of *cwg1-1*. In ensuing experiments, total or partial 5′ or 3′-end deletions or simple deletion of the ATG start codon of *bgs4*$^{pbr1-8}$ ORF suppressed both *bgs4*$^{pbr1-8}$ function and antifungal resistance. These results therefore indicate that *pbr1-8* resistance is only due to the *bgs4*$^{pbr1-8}$ mutation.

The expression level of bgs4^{+} *determines its capacity to suppress the pbr1-8 resistance phenotype.* In order to study the unexpected effect of the absence of *pbr1-8* resistance suppression by *bgs4*$^{+}$, a protocol for micro-culture assays was developed. As before, heterozygous WT / *pbr1-8* showed the recessive resistance trait and p*bgs4*$^{+}$ was unable to suppress the haploid *pbr1-8* or diploid *pbr1-8* / *pbr1-8* resistances, except to caspofungin (Table 3). The *pbr1-6* strain showed resistance to the four drugs and in this case *bgs4*$^{+}$ was able to suppress the four resistances (Table 3).

However, when the wild-type *bgs4*$^{+}$ and mutant *bgs4*$^{pbr1-8}$ plasmids were expressed in the same cell, either haploid or diploid, in the absence of endogenous *bgs4* gene p*bgs4*$^{+}$ was able to suppress the resistance due to p*bgs4*$^{pbr1-8}$ (Table 4). Therefore, *bgs4*$^{+}$ expressed from a plasmid is unable to suppress the endogenous *pbr1-8* resistance but is able to suppress a similar resistance conferred by the *bgs4*$^{pbr1-8}$ plasmid.

The absence of *pbr1-8* suppression by p*bgs4*$^{+}$ was opposite to the suppression observed with p81X-*bgs4*$^{+}$ (Fig. 6C). To study this discrepancy, p81X-*bgs4*$^{+}$ was analyzed in the *pbr1-8* and *pbr1-8* / *pbr1-8* strains that had previously showed an absence of suppression by p*bgs4*$^{+}$ (Table 3). Surprisingly, induced p81X-*bgs4*$^{+}$ was able to suppress the resistance of both strains (Table 5). This suggests that the ability of *bgs4*$^{+}$ to suppress the *pbr1-8* resistance depends on the expression level of the corresponding *bgs4*$^{+}$ plasmid. This was confirmed by expressing p*bgs4*$^{+}$ in hemi-dosage *pbr1-8* / *bgs4*Δ diploid cells. Whereas p*bgs4*$^{+}$ did not suppress *pbr1-8* / *pbr1-8* resistance (Table 3), it did suppress pbr1-*8* / *bgs4*Δ resistance (Table 5). This shows that the ectopic gene expression may be less efficient than the chromosomal gene expression, sufficient to suppress some (*cwg1-1*, *cwg1-2*, *orb11-56*, *pbr1-6*) but not other mutations (*pbr1-8*).

DISCUSSION

S. pombe is an attractive model to study the effect of antifungals specific to β(1,3)glucan synthesis. Its special rod shape makes the fission yeast an excellent organism to explore

morphological defects. Any minor cell wall defect will result in detectable morphological changes. In addition, *S. pombe* has no detectable chitin and therefore β(1,3)glucan acquires more relevance as a structural cell wall polysaccharide. Most fungi contain a single essential Fks protein or two redundant Fks subunits, which are only essential when both are affected. In this sense, *S. pombe* is also special since it contains four Fks homologs, Bgs1 to Bgs4, and three of them - Bgs1, Bgs3 and Bgs4- are essential for vegetative cells. Therefore, study of the effect of GS inhibitors on *S. pombe* cells, GS activity, and Bgs subunits could provide important information about the mechanism of action of each family of specific GS inhibitors.

Analysis of the resistance of fission yeast to GS inhibitors could also provide interesting information. Resistance to GS inhibitors in other fungi is associated with mutations in their single Fks protein. In the case of *S. pombe*, a mutation in only one of the three essential Bgs proteins would not alter the antifungal sensitivity of the two other Bgs subunits and the cell would remain sensitive to the antifungals. Therefore, which mechanism confers antifungal resistance to *S. pombe* cells? Initially, one possibility was that the resistance was due to a general regulator of the three Bgs proteins. However, cloning of the gene involved in the resistance revealed that it was exclusively associated with Bgs4, implying that Bgs1 and Bgs3 are not affected by the antifungals. This opens interesting new questions about the intrinsic resistance of Bgs1 and Bgs3, even though their sequences harbor all the residues that are determinant for the Bgs4 / Fks sensitivity to GS antifungals. The identification of Bgs1 and Bgs3 as new antifungal targets opens new possibilities in the search for new GS inhibitors with a broader mechanism of action.

There are several antifungal families that specifically inhibit β(1,3)glucan synthesis and GS activity (2,12,44,45), but little is known about their mechanisms of action. In this work, we compared the effect of three GS inhibitor families on *S. pombe* wild-type and papulacandin-resistant cells and GS activities, and we found interesting differences both *in vivo* and *in vitro*. In wild-type cells, papulacandin and enfumafungin cause generalized cell lysis, mainly during cell separation (our unpublished results) and subsequent total cell growth arrest, whereas resistant cells are not affected. Echinocandins are different; the lysis of wild-type cells is incomplete and the surviving cells become rounded and maintain a residual cell growth. The resistant cells are partially affected in morphology and cell growth and their resistance is variable, depending on the type of mutant and echinocandin, ranging from high (*pbr1-8* with pneumocandin), to low (caspofungin), to non-resistant (*pbr1-6* with aculeacin). This effect could be due to a distinct permeability to the different drugs, but in that case the cell should present similar or higher resistance than its *in vitro* GS resistance, and in our case we observed the opposite. It should be noted that whereas *pbr1-8* and *pbr1-6* were highly resistant to papulacandin and enfumafungin, they exhibited the opposite behavior with respect to aculeacin; *pbr1-8* was highly resistant and *pbr1-6* was sensitive. The finding of this variability in the resistance to echinocandins was possible because the mutants were selected for their resistance to papulacandin (25). Most of the mutants described have been isolated as resistant to echinocandins (12,21,22,37-43). We have inserted some of these mutations into $bgs4^{+}$, and none of them altered the *in vivo* sensitivity of the cells to papulacandin and enfumafungin, but produced variable degrees of resistance to echinocandins (our unpublished results), showing that each antifungal family acts through different sites of the GS enzyme and that some resistances may be specific to a given antifungal family. We have also analyzed the GS inhibitor Aerothricin3 / FR901469 (generous gift from Dr. Osamu Kondoh, Chugai Pharmaceutical) (46) on wild-type and resistant cells and we found no differences. GS antifungals such as Aerothricin3, acting on different sites of the Bgs4 / Fks proteins, could be good candidates for a combined therapy to minimize the appearance of resistant strains.

The MIC values of papulacandin and aculeacin described for *S. cerevisiae* and *Candida* are similar to the values for *S. pombe* (11,25,47,48). In other cases, the MIC value of a specific inhibitor fluctuate, depending on the method of analysis, the strain, or the organism. The MIC values of caspofungin described for *C. albicans* (0.0125-0.8 μg/ml) and other *Candida* species (0.01-8.0 μg/ml) are highly variable, although overall they denote a lower MIC compared to that obtained for *S. pombe* (11,23,49). This could be due to a lower susceptibility of Bgs4 or to a compensatory mechanism of the intrinsic resistant Bgs1 and Bgs3 subunits.

We compared the *in vitro* effect of each antifungal family on wild-type GS activity. Papulacandin exerted an *in vitro* effect that was 10^3-10^4-fold stronger than that of the other drugs, except caspofungin. This effect has not been described in previous studies (25), probably due to the method used to prepare the GS enzyme. However, this *in vitro* difference was not correlated with a similar increase in *in vivo* susceptibility. This could be due to other antifungal interactions with the cell or to the *in vivo* accessibility of the enzyme. A similar case was observed with caspofungin, with an IC_{50} 10^2-10^3-fold lower than that of the other drugs, except papulacandin, with no correlation with its *in vivo* inhibitory capacity. By contrast, some *in vitro* mutant GS resistances to pneumocandin, aculeacin or caspofungin are much higher than the resistances detected in the cells. Furthermore, some *bgs4* mutants are not resistant *in vivo* but do show *in vitro* GS resistance to echinocandins (our unpublished results). These differences show that *in vitro* GS resistance may be insufficient to confer resistance to cells, and suggest the presence of other targets modulating the GS activity *in vivo*.

Interestingly, we observed that some of the antifungals tested were able to increase the mutant GS activity above the initial activity. This activating effect was observed in *pbr1-8* but not in *pbr1-6* GS activity, denoting specific and antagonistic drug effects that depend on the interacting GS conformation. *pbr1-8* is activated by enfumafungin and echinocandins but other *bgs4* mutants are also activated by papulacandins or only one antifungal (our unpublished results). No GS activation by GS inhibitors has been reported, except for papulacandin (50). In that case, the drug activation affected the wild-type GS and was dependent on low substrate concentrations. It is possible that some mutations, such as that of *pbr1-8*, in the presence of an antifungal could mimic the proposed preferential binding of substrate to the active form of the enzyme.

Among the inhibitors studied, caspofungin was seen to be the best candidate for antifungal therapy. The caspofungin MIC of resistant strains was much lower than that of the other drugs and it produced a reduction in cell growth and an altered morphology of resistant cells. Caspofungin also showed special properties as regards the *in vitro* GS activity, with an IC_{50} 10^2-10^3-fold lower than that of other drugs, except papulacandin, and with two hitherto unreported inhibitory effects of high- (<0.7 μg/ml) and low- (>70 μg/ml) affinity. This suggests the presence of two GS interaction sites with caspofungin. Both *pbr1-8* and *pbr1-6* GS were resistant to the high-affinity inhibition but not to the low-affinity inhibition, suggesting that the resistance hot spots 1-1 and 1-2 would be located in the region involved in this high-affinity interaction with caspofungin. One candidate for the low-affinity interaction site could be a short hot spot 2 located in the predicted second transmembrane region (12,41). Alternatively, the low-affinity inhibition could be due to interactions between caspofungin and Bgs1 or Bgs3. Both hypotheses are currently under investigation.

The interesting effect of caspofungin in that it produces two separate inhibitory effects is different from the paradoxical growth effect, or Eagle effect, described for *Candida* (51,52), which consists of an *in vivo* attenuation of growth inhibition at drug concentrations above the inhibitory concentration. The result is growth inhibition followed by a resumption of growth at higher antifungal concentrations and a new inhibitory effect when the drug concentration increases. This phenomenon has been associated with compensatory mechanisms from the cell integrity and calcineurin pathways. In our case, the effect was observed in the GS activity *in vitro,* and therefore it cannot be explained in terms of cell compensatory mechanisms. In addition, the GS activity did not increase but remained constant over a broad concentration range of antifungal until a new inhibition appeared.

All the mutants selected as resistant to echinocandins have been described as dominant or semi-dominant (12,20,33,34,36,37,53). Only mutants isolated as resistant to papulacandin have shown a recessive trait (25). *S. cerevisiae pbr1-1* resistance is suppressed by the *PBR1* / *FKS1* gene. However, although *S. pombe pbr1-8* resistance was clearly recessive and caused by a $bgs4^+$ mutation, $bgs4^+$ expressed from plasmids was unable to suppress it. Our studies show that chromosomal and plasmid expression may differ, at least in their resulting phenotype; a recessive chromosomal trait can appear as dominant when the complementing gene is expressed ectopically. However, the complementing gene remains dominant over the recessive allele when both are expressed ectopically or when the chromosomal recessive gene dosage decreases, as is the case of hemi-dosage diploid cells. This explains the ability of $bgs4^+$ plasmids to suppress -in *pbr1-8*

cells- the low resistance to caspofungin, but not higher resistances to the other inhibitors. These results show that in some cases typical gene cloning by suppression of the recessive mutant phenotype may not be possible and hence other techniques must be used.

All the echinocandin resistance mutations have been localized in a 9-amino-acid resistance hot spot 1. In this work, we found interesting new mutations that conferred resistance to the three antifungal families and were external to the hot spot 1. One of these mutations extends the hot spot to 13 amino acids in the resistance hot spot 1-1. The other mutation, although in the same region, is distant and defines a new resistance hot spot 1-2. The use of other GS-specific drugs, such as papulacandin, and the use of other species, such as *S. pombe,* have been shown to be helpful to define new amino acids and regions in GS that are important for interaction with antifungals. Studying these and other new resistances will help to develop more efficient antifungals, such as caspofungin, which is able to inhibit resistant mutants at concentrations closer to those effective for wild-type cells.

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FOOTNOTES

The abbreviations used are: GS, β(1,3)glucan synthase; MIC, minimal inhibitory concentration; IC_{50}, half-maximal inhibitory concentration.

FIGURE LEGENDS

Fig. 1. Morphology of *pbr1-8* and *pbr1-6* cells. (A) Normal and lemon shaped morphology of *pbr1-8* and *bgs4*Δ *bgs4*$^{pbr1-8}$ cells and rounded morphology of *pbr1-6* and *bgs4*Δ *bgs4*$^{pbr1-6}$ cells. Osmotic stabilization with sorbitol (S) suppresses the *pbr1-6* rounded morphology, after which a multiseptated phenotype appears. Phase-contrast and Calcofluor white-stained (50 μg/ml) micrographs of early log-phase cells grown on YES medium at 28 °C. Bar, 10 μm. (B) Spherical morphology of original *pbr1-6* strain (W760S substitution) and elongated cell-shape of a spontaneous *pbr1-6* morphological revertant (W760S and A914V changes) and of a *bgs4*Δ *bgs4*$^{pbr1-6\ (W760S\ A914V)}$ strain. The *pbr1-6* resistance is not affected with the A914V suppressor mutation (Table 3).

Fig. 2. Chemical structures of the three families of specific β(1,3)glucan synthase inhibitors. Structures of the inhibitors representative of each family used in this work: papulacandin B (papulacandins), enfumafungin (acidic terpenoids), aculeacin A, pneumocandin B0 and caspofungin (echinocandins).

Fig. 3. Differential effect of papulacandin B and enfumafungin and of pneumocandin B0, aculeacin A and caspofungin on the cell growth of the wild-type, *pbr1-8*, and *pbr1-6* strains. Early log-phase cells were grown in YES medium at 28ºC either in the absence or in the presence of the indicated antifungal concentrations. Cell growth was monitored at the indicated times. (A) papulacandin and enfumafungin produce total cell growth arrest in wild-type cells whereas *pbr1-8* cells are completely resistant. However, echinocandins permit a residual growth of wild-type cells and produce slow cell growth in *pbr1-8* cells. (B) *pbr1-6* cells are resistant to only some antifungals; very resistant to papulacandin; partially resistant to pneumocandin, and sensitive to aculeacin. *pbr1-6* cells are also resistant to enfumafungin and caspofungin (see Tables 2 and 3). Pap, papulacandin. Enf, enfumafungin. Pne, pneumocandin. Acu, aculeacin. Csp, caspofungin.

Fig. 4. Differential inhibitory effect of papulacandin B, enfumafungin, pneumocandin B0, aculeacin A and caspofungin on the *in vitro* β(1,3)glucan synthase (GS) activity of wild-type and *pbr1* cell extracts. (A) Inhibitory effect on the wild-type GS activity. Papulacandin shows an inhibitory capacity 10^3-10^4-fold higher than the other antifungals, except caspofungin (see Table 2). (B) Inhibitory effect on the GS activity of *pbr1* cell extracts. *pbr1-6* and *pbr1-8* GS are highly resistant, with IC_{50} values above 250 μg/ml. The *pbr1-6* strain was characterized *in vivo* as aculeacin A-sensitive but its *in vitro* GS activity was partially resistant. (C) Inhibitory effect of caspofungin on the wild-type GS activity. Caspofungin exhibits a very high inhibitory capacity: 10^2-10^3-fold higher than other antifungals, except papulacandin (see Table 2). Caspofungin shows a dual inhibitory effect, at low and high concentrations (first and third sections marked by dotted lines), separated by a plateau of a 100-fold drug increase with no increase in inhibition (middle section marked by dotted lines). (D) Resistance of *pbr1-6* and *pbr1-8* GS activities to caspofungin. *pbr1-8* GS is totally resistant and *pbr1-6* is partially resistant to the low-concentration inhibitory effect, but both GS are sensitive to the high-concentration inhibitory effect. Caspofungin is the only antifungal able to produce a noticeable inhibition of both mutant GS, with IC_{50} values below 250 μg/ml. Results are shown as percentages of residual GS activity. Error bars show standard deviations.

Fig. 5. The resistance phenotypes of *pbr1-6*, but not those of *pbr1-8*, can be suppressed by *bgs4*$^+$ expression. (A) *bgs4*$^+$ expression cannot suppress the recessive resistances to papulacandin, enfumafungin and pneumocandin of *pbr1-8*. *bgs4*$^+$ is only able to suppress the caspofungin resistance of *pbr1-8*. Cells were grown and monitored as in fig 3. (B) The recessive *pbr1-6* resistances to GS antifungals are suppressed by *bgs4*$^+$ expression (see also Table 3). (C) *bgs4*$^+$ is unable to suppress the *in vitro pbr1-8* GS resistance. Extracts of cells transformed with empty or *bgs4*$^+$ multicopy plasmids

were assayed for *in vitro* GS activity in the presence of 10 μg/ml of papulacandin or 60 μg/ml of pneumocandin (concentrations that produce high inhibition of the wild-type GS) as in fig. 4.

Fig. 6. *bgs4* is responsible for at least part of the resistance phenotype of *pbr1-8* strain. Cells were grown and monitored as in fig 3. (A) *bgs4* cloned from the *pbr1-8* strain confers antifungal resistance to a *bgs4*Δ strain. The $bgs4^{pbr1\text{-}8}$ resistance is recessive in a wild-type ($bgs4^{+}$) strain. (B) The *pbr1-8* resistance is recessive in heterozygosis (WT / *pbr1-8*). However, *pbr1-8* confers resistance to *pbr1-8* / *bgs4*Δ diploid strains. (C) The *pbr1-8* / *bgs4*Δ resistance phenotype can be suppressed by $bgs4^{+}$ expression. The expression of p81X-$bgs4^{+}$ suppresses the *pbr1-8* / *bgs4*Δ resistance when the 81X promoter is induced (- thiamine, -T) and maintains the *pbr1-8* / *bgs4*Δ resistance when the 81X promoter is repressed (+ thiamine, +T).

Fig 7. *pbr1-6* and *pbr1-8* resistances are due to single amino acid substitutions of the Bgs4 sequence. (A) Hydropathy profile of Bgs4. The two predicted TM regions and the sites where $Bgs4^{pbr1\text{-}8}$-E700V and $Bgs4^{pbr1\text{-}6}$-W760S are located in the first TM region are shown. (B) Sequence alignment of a conserved region of 70 amino acids of Bgs1, Bgs2, Bgs3 and Bgs4 from *S. pombe*, Fks1 and Fks2 from *S. cerevisiae* (Sc), Gsc1 (Fks1) from *Candida albicans* (Ca), and Fks1 and Fks2 from *Candida glabrata* (Cg). The amino acid mutations described to confer resistance to echinocandins in Sc, Ca and Cg, defining a resistance hot spot 1 of 9 amino acids, are shown. The $Bgs4^{pbr1\text{-}8}$ mutation is located 4 amino acids N-terminal from hot spot 1, increasing the cluster to a 13 amino acid hot spot 1-1 of resistance to papulacandin, enfumafungin and echinocandins. The $Bgs4^{pbr1\text{-}6}$ change is located 48 amino acids C-terminal from hot spot 1-1, defining a novel hot spot 1-2 of resistance to the three antifungal families.

TABLE 1. Increasing cell wall defects of *S. pombe pbr1-8* and *pbr1-6* strains.

Strain	Alkali extraction[a]	% Lysis of cell suspensions[b]		% Incorporation of [^{14}C]glucose[c]				Cell wall hexoses[d]		
	% residual cell wall	Novozyme	Zymolyase	Cell wall	β-Glucan	α-Glucan	Galactomannan	Glc	Man	Gal
WT	12.7 ± 1.6	13.4 ± 3.3	71.7 ± 3.0	25.4 ± 0.3	14.0 ± 0.2	7.6 ± 0.4	3.8 ± 0.3	88.8 ± 0.1	7.4 ± 0.2	3.7 ± 0.2
pbr1-8	10.9 ± 0.6	50.1 ± 6.5	60.9 ± 2.6	27.7 ± 0.7	15.1 ± 0.5	9.2 ± 0.5	3.4 ± 0.1	91.3 ± 1.0	5.5 ± 0.6	3.2 ± 0.3
pbr1-6	1.8 ± 0.2	69.6 ± 4.1	44.1 ± 3.9	28.2 ± 1.0	15.2 ± 0.9	11.7 ± 0.8	1.3 ± 0.2	97.4 ± 0.6	1.8 ± 0.3	0.7 ± 0.2

Values are the means and standard deviations (SD) calculated from at least three independent experiments.

[a] Mild alkali extraction of the cell wall (6% NaOH, room temperature, 5 min). Standard alkali extraction removes the entire cell wall.

[b] Percentage of OD_{600} decrease after 6 hours in the presence of Novozyme (10 mg/ml) or Zymolyase (2 mg/ml). Novozyme 234 complex degrades the entire cell wall whereas Zymolyase 100T degrades all the cell wall except α-glucan.

[c] Percentage incorporation of [^{14}C]glucose into the cell wall polysaccharides (cpm incorporated per fraction x 100 / total cpm incorporated).

[d] Percentage of cell wall hexoses analyzed by gas-liquid chromatography.

Table 2. *In* vivo Minimal Inhibitory Concentration (MIC) and *in vitro* half-maximal inhibitory concentration (IC_{50}) of wild type, *pbr1-8* and *pbr1-6* strains.

MIC (µg/ml)				
Strain	**Papulacandin**	**Enfumafungin**	**Pneumocandin**	**Caspofungin**
WT	5	10	5	10
pbr1-8	>100	>100	>100	50
pbr1-6	>100	>100	50	30

IC_{50} (µg/ml)				
Strain	**Papulacandin**	**Enfumafungin**	**Pneumocandin**	**Caspofungin**
WT	0.02	40	120	0.3
pbr1-8	>250	>250	>250	250
pbr1-6	>250	>250	>250	150

Table 3. *bgs4*$^{+}$ plasmids cannot suppress the recessive resistance mutation of the *pbr1-8* strain but are able to suppress that of the *pbr1-6* strain.

Strain	Papulacandin (20 μg/ml)	Enfumafungin (20 μg/ml)	Echinocandin (20 μg/ml)	Caspofungin (20 μg/ml)
WT	-	-	-	±
WT (pJK-*bgs4*$^{pbr1-8}$)	±	±	±	±
WT pAL-*bgs4*$^{pbr1-8}$	±	±	±	±
pbr1-8	+++	+++	+++	+++
pbr1-8 (pJK-*bgs4*$^{+}$)	+++	+++	++	+
pbr1-8 pAL-*bgs4*$^{+}$	+++	+++	++	+
WT / WT	-	-	-	-
pbr1-8 / *pbr1-8*	+++	+++	+++	+++
WT / *pbr1-8*	±	±	±	-
WT / WT (pJK-*bgs4*$^{pbr1-8}$)	-	-	±	-
WT / WT pAL-*bgs4*$^{pbr1-8}$	±	-	±	-
pbr1-8 / *pbr1-8* (pJK-*bgs4*$^{+}$)	+++	+++	+++	+
pbr1-8 / *pbr1-8* pAL-*bgs4*$^{+}$	+++	+++	+++	+
pbr1-6 (W760S)	+++	+++	+++	++
pbr1-6 (W760S A914V)	+++	+++	+++	++
pbr1-6 / *pbr1-6*	+++	+++	+++	+++
WT / *pbr1-6*	-	-	-	-
WT (pJK-*bgs4*$^{pbr1-6}$)	±	±	±	-
WT pAL-*bgs4*$^{pbr1-6}$	±	±	±	-
pbr1-6 (pJK-*bgs4*$^{+}$)	+	±	±	±
pbr1-6 pAL-*bgs4*$^{+}$	+	±	±	±

(pJK-*bgs4*$^{+}$): single copy integrative plasmid; pAL-*bgs4*$^{+}$: multicopy plasmid.
The *pbr1-6* (W760S A914V) strain contains the spontaneous A914V substitution that suppresses the spherical morphology but maintains the resistance phenotype of *pbr1-6* (W760S) strain.
-: no growth; +++: total growth.

Table 4. The resistances conferred by *bgs4*$^{pbr1-8}$ plasmids to a *bgs4*Δ strain can be suppressed by *bgs4*$^{+}$ plasmids.

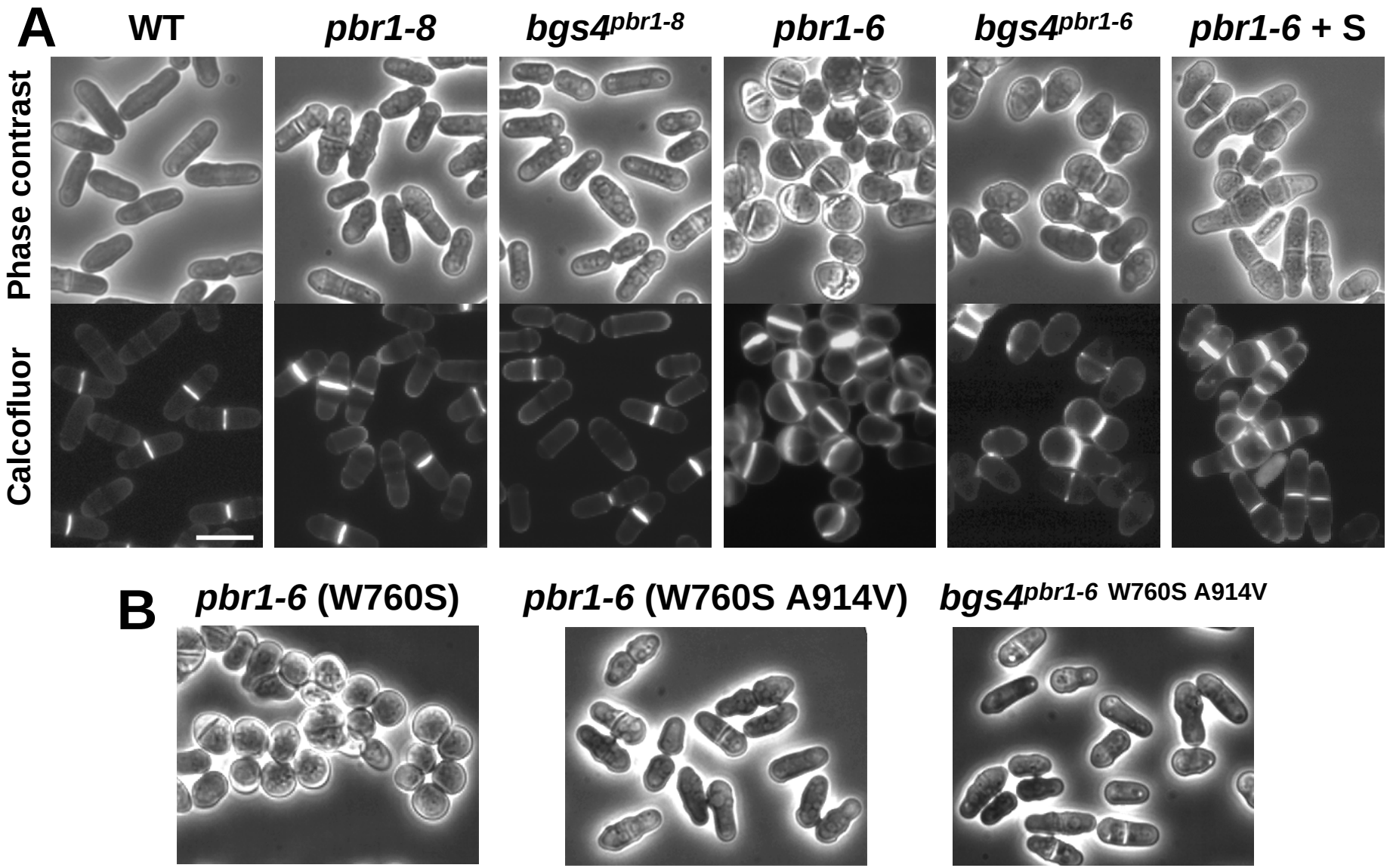
Strain	Papulacandin (20 μg/ml)	Enfumafungin (20 μg/ml)	Echinocandin (20 μg/ml)	Caspofungin (20 μg/ml)
*bgs4*Δ S4	-	-	-	±
*bgs4*Δ SH	-	-	-	±
*bgs4*Δ R4	+++	+++	+++	++
*bgs4*Δ RH	+++	+++	+++	++
*bgs4*Δ S4 SH	-	-	-	±
*bgs4*Δ R4 RH	+++	+++	+++	+++
*bgs4*Δ S4 RH	-	-	-	-
*bgs4*Δ R4 SH	±	±	-	-
*bgs4*Δ S4 / *bgs4*Δ SH	-	-	-	-
*bgs4*Δ R4 / *bgs4*Δ RH	+++	+++	+++	+++
*bgs4*Δ S4 / *bgs4*Δ RH	-	-	-	-
*bgs4*Δ R4 / *bgs4*Δ SH	-	-	-	-

S4: pJK-*bgs4*$^{+}$ inserted into the *bgs4*$^{+}$ promoter.
R4: pJK-*bgs4*$^{pbr1-8}$ inserted into the *bgs4*$^{+}$ promoter.
SH: phis3-*bgs4*$^{+}$ inserted into the *his3*$^{+}$ promoter.
RH: phis3-*bgs4*$^{pbr1-8}$ inserted into the *his3*$^{+}$ promoter.
-: no growth; +++: total growth.

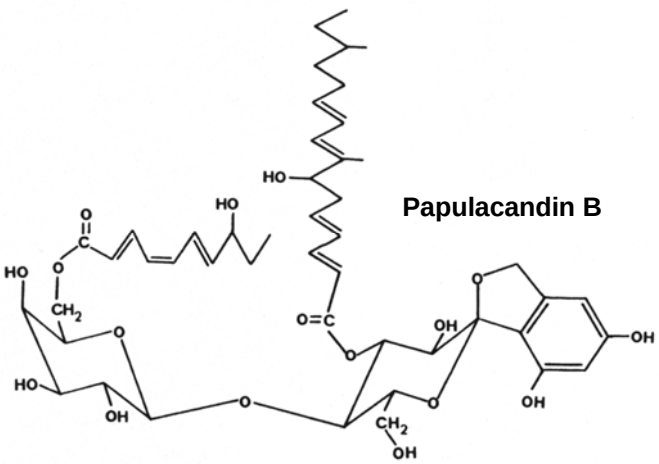
Table 5. Suppression of *pbr1-8* resistances by regulatable *bgs4*$^{+}$ expression plasmid and of hemi-dosage *pbr1-8* / *bgs4*Δ diploid resistances by *bgs4*$^{+}$ plasmids (own promoter) suggests a dosage-dependent suppression (compare with Table 3).

Strain	Papulacandin (20 μg/ml)	Enfumafungin (20 μg/ml)	Echinocandin (20 μg/ml)	Caspofungin (20 μg/ml)
WT p81X-*bgs4*$^{pbr1-8}$ -T (on)	-	-	-	-
WT p81X-*bgs4*$^{pbr1-8}$ +T (off)	-	-	-	-
pbr1-8 p81X-*bgs4*$^{+}$ -T (on)	-	±	±	-
pbr1-8 p81X-*bgs4*$^{+}$ +T (off)	+++	+++	+++	++
WT / WT p81X-*bgs4*$^{pbr1-8}$ -T (on)	-	-	-	-
WT / WT p81X-*bgs4*$^{pbr1-8}$ +T (off)	-	-	-	-
pbr1-8 / *pbr1-8* p81X-*bgs4*$^{+}$ -T (on)	-	±	-	±
pbr1-8 / *pbr1-8* p81X-*bgs4*$^{+}$ +T (off)	+++	+++	+++	++
WT / *bgs4*Δ	-	-	-	-
pbr1-8 / *bgs4*Δ	+++	+++	+++	+++
WT / *bgs4*Δ (pJK-*bgs4*$^{pbr1-8}$)	±	±	±	±
WT / *bgs4*Δ pAL-*bgs4*$^{pbr1-8}$	±	±	-	-
pbr1-8 / *bgs4*Δ (pJK-*bgs4*$^{+}$)	±	±	±	±
pbr1-8 / *bgs4*Δ pAL-*bgs4*$^{+}$	±	±	-	-

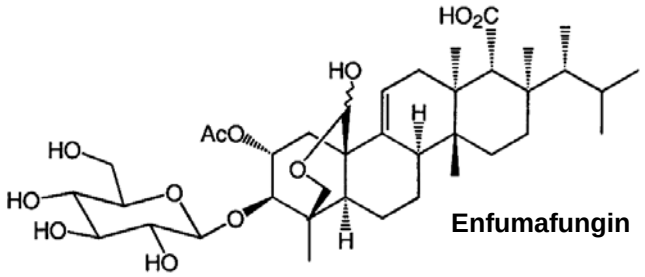
p81X-*bgs4*$^{+}$: multicopy plasmid with the 81X version of the thiamine repressible *nmt1*$^{+}$ promoter.
-T: absence of thiamine, induced conditions (on). +T: presence of thiamine, repressed conditions (off).
(pJK-*bgs4*$^{+}$): single copy integrative plasmid; pAL-*bgs4*$^{+}$: multicopy plasmid.
-: no growth; +++: total growth.


A
WT
pbr1-8
bgs4pbr1-8
pbr1-6
bgs4pbr1-6
pbr1-6 + S
Phase contrast
Calcofluor
B
pbr1-6 (W760S)
pbr1-6 (W760S A914V)
bgs4pbr1-6 W760S A914V

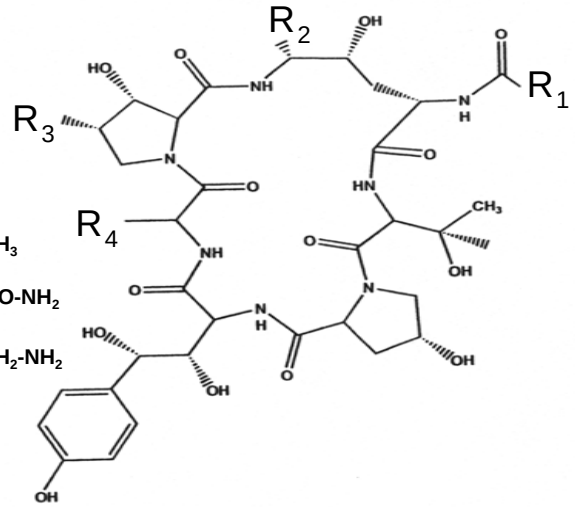
Martins *el al.* Fig 2



Papulacandin B

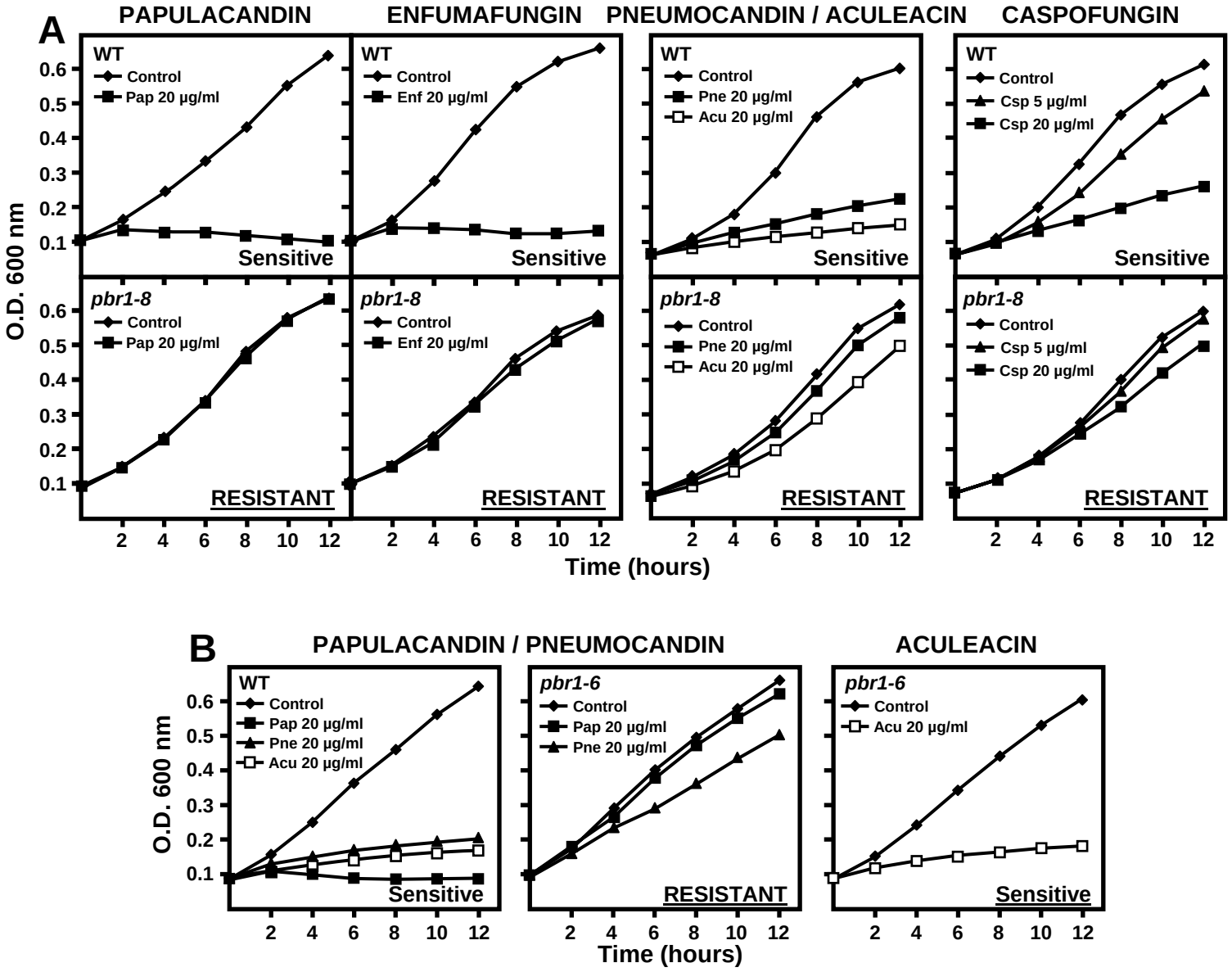


Enfumafungin

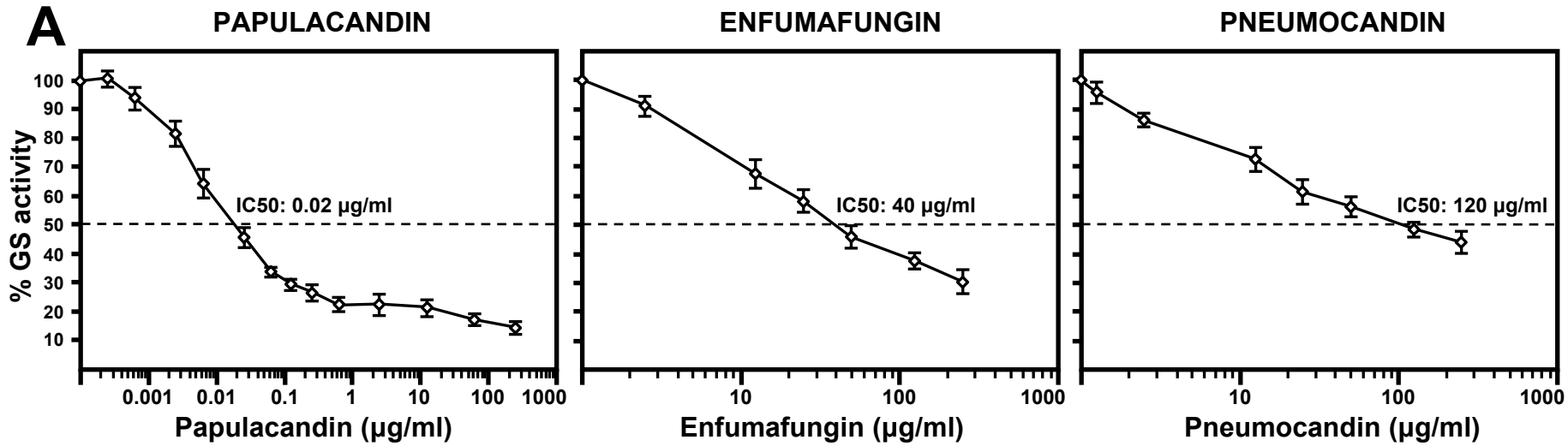


	R_1	R_2	R_3	R_4
Aculeacin A		OH	CH_3	CHOH-CH_3
Pneumocandin B0 (L-688786)		OH	H	CHOH-CH_2-CO-NH_2
Caspofungin (Cancidas, L-743872, MK-0991)		NH-CH_2-CH_2-NH_2	H	CHOH-CH_2-CH_2-NH_2

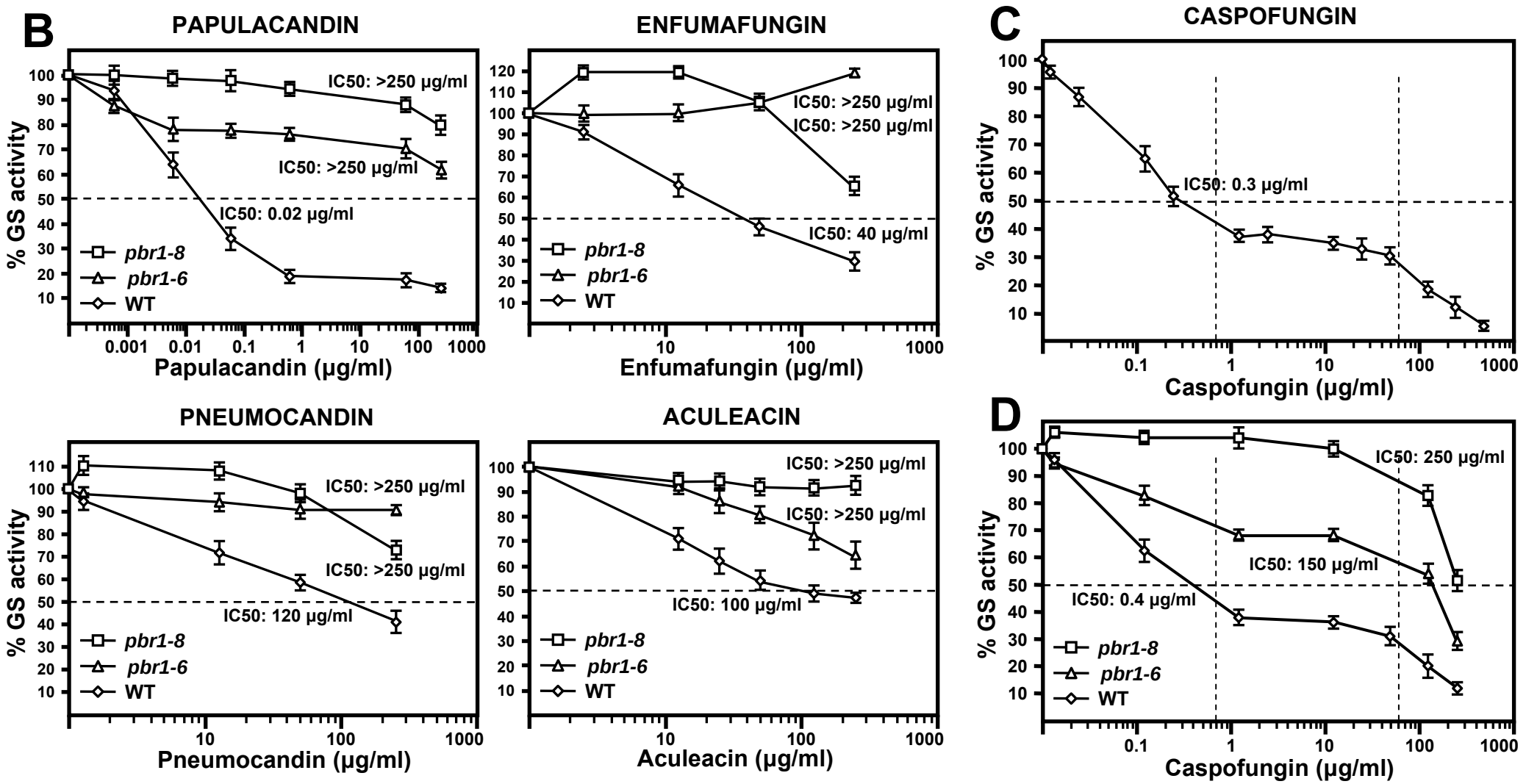
Martins *el al.* Fig 3



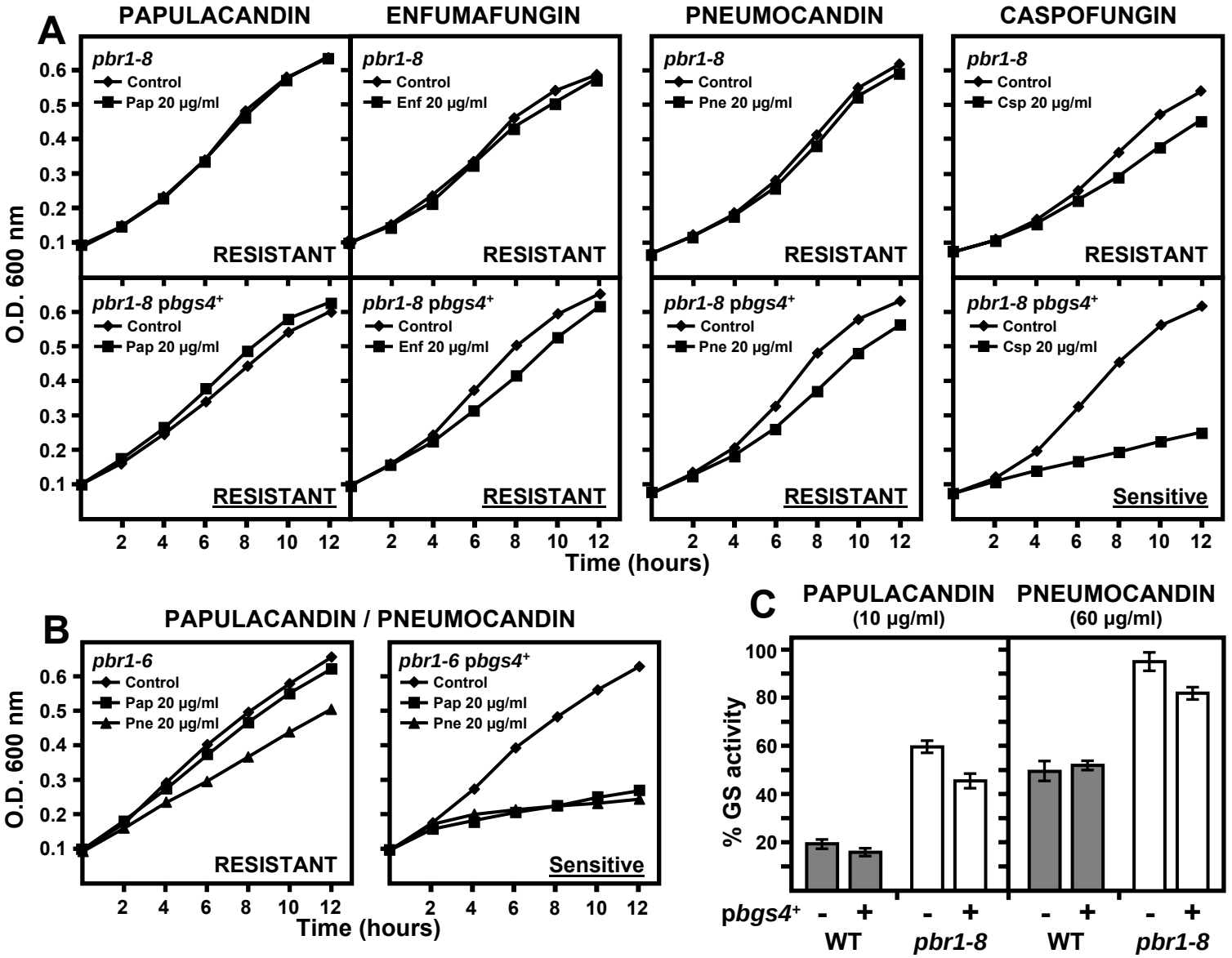
Martins *el al.* Fig 4A



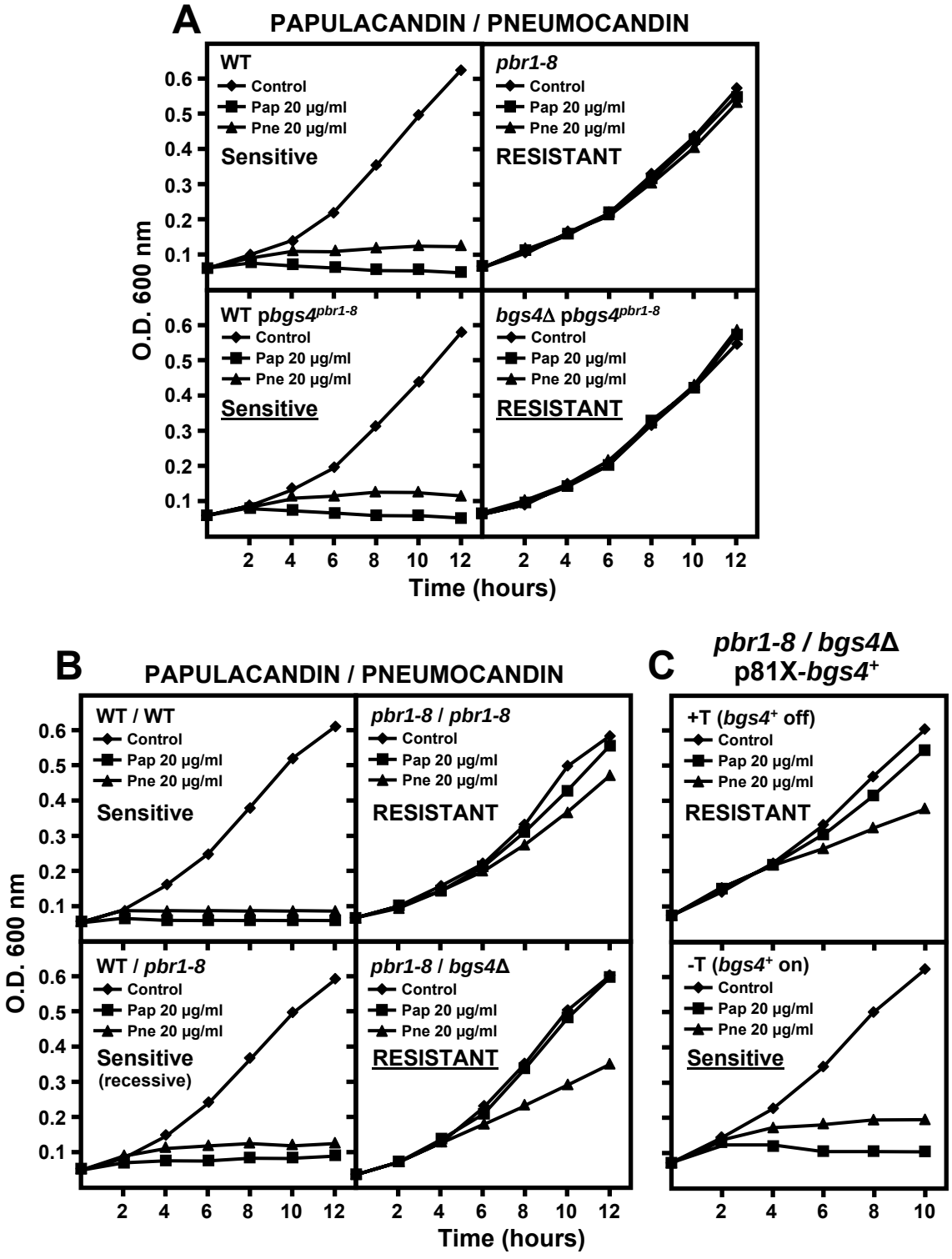
Martins *el al.* Fig 4B



Martins *el al.* Fig 5



Martins *el al.* Fig 6



Martins *el al.* Fig 7